Contribution of Chlorination to the Mutagenic Activity of Drinking Water Extracts in Salmonella and Chinese Hamster Ovary Cells

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The production of chlorinated by-products through chlorine disinfection of drinking water has been well documented. Natural organic precursors for these chemicals include fulvic and humic acids, the chlorination of which leads to the production of mutagenic compounds. Comparisons of extracts of raw versus treated waters have confirmed that clorination during water treatment produces mutagenic activity in the Salmonella (Ames) test. Present work on XAD-2 extracts of raw and chlorinated water from six municipalities in the Great Lakes region of Canada has involved a battery of mutagenicity assays for various genetic endpoints: the Salmonella test, the sister-chromatid exchange (SCE), and the micronucleus (MN) induction in Chinese hamster ovary (CHO) cells. All extracts of treated (chlorinated), but none of untreated, water were mutagenic in the Salmonella assay. On the other hand, extracts of both treated and untreated water samples showed activity in the SCE and MN assays, but no consistent pattern of response with regard to treatment (chlorination) was evident. These data show that chlorination contributes mutagens to drinking water and suggest that mammalian in vitro assays may be more sensitive for detecting mutagenicity in water samples than the Salmonella test.

Introduction

It is a well established principle that organic compounds in chlorinated drinking water, from a number of sources, are mutagenic. This activity has, for the most part, been observed in bacteria using the Salmonella/mammalian microsome assay to test concentrates of nonvolatile constituents (1-3). A portion of this mutagenicity has been attributed to the reaction of chlorine used in the disinfection process with pre-existing organic precursors present in untreated source water (4-6).

One of the principal reasons for studying the mutagenicity of drinking water extracts has been the well established association between the phenomena of mutagenicity and carcinogenicity (7). It is possible to perform carcinogenicity studies on individual drinking water extracts in rodents, and such studies have yielded

both positive (8,9) and negative (10) findings. However, such assays are not amenable to the study of samples from a number of sources because of the time necessary for these costly long-term studies and also because of the large quantities of material that are required. Accordingly, mutagenicity assays have been expected to serve as suitable, easily performed surrogates for carcinogenicity studies and as indicators of possible induced heritable mutations in these cases. However, in spite of the fairly universal finding that drinking water concentrates are mutagenic, there is no adequate interpretation of the relevance of the level of drinking water mutagenicity in terms of hazard to human beings. Part of this problem is caused by the fact that most studies have tested bacteria, organisms that are convenient to use when conducting experiments, but whose reactions may not be relevant to human health. To establish a more relevant basis for the prediction of human health hazard, most testing strategies include, as a minimum, a test for in vitro cytogenetic effects in mammalian cells, in addition to a test for microbial gene mutation (11). In keeping with this concept, the present study was undertaken to relate chlorination-associated mutagenic effects in Salmonella to mammalian cell genotoxicity to establish a more relevant basis for the prediction of hazard.

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In this study, samples of raw and chlorinated drinking water from six municipalities in the Great Lakes region of the Province of Ontario, Canada, were concentrated using XAD-2 resin. Extracts of organics so derived were tested for the induction of gene mutation using the Salmonella/mammalian microsome assay and for the induction of micronuclei (MN) (chromosomal aberrations) and sister-chromatid exchanges (SCE) in cultured Chinese hamster ovary (CHO) cells.

Materials and Methods

Water Sampling

Stainless steel, 3.2 cm × 40 cm cartridges were packed with XAD-2 Amberlite resin as described previously (12). Pairs of cartridges were installed in parallel by connecting them to a supply tap, and a maximum of approximately 1450 L of water was passed through each cartridge at a rate of approximately 1 L/min. Following this procedure, columns were capped and transported to the laboratory for elution of adsorbed material using 1 L of a hexane:acetone (85:15) mixture and concentration in a rotary evaporator (12). The resulting concentrates were evaporated either to dryness (dry extracts) or retained as they were (wet extracts) for mutagenicity testing. To determine if the solvents used could produce artifactual results, a freshly prepared cartridge was eluted with the hexane/acetone mixture without carrying out the sampling procedure. In all cases, glass-distilled, pesticide-grade solvents were used.

Salmonella/Mammalian Microsome Assay

Both dry and wet extracts were tested for reverse mutation in Salmonella strains TA 98 and TA 100, both with and without the addition of Aroclor 1254-induced, Sprague-Dawley rat liver S9 mix (13). These strains were selected because in the past they have consistently given the optimum responses to drinking water extracts (1). Dry extracts were dissolved in acetone before testing, and wet extracts were added directly to the top-agar mixture. The criteria for a positive response were a doubling over background with at least one treatment and a dose-response as evidenced by dose-related increases over the background at a minimum of two treatment points.

Chinese Hamster Ovary Assays

Attempts to test wet extracts for the induction of SCE and MN in CHO cells were unsuccessful because of the immiscibility of the hexane/acetone mixture in the culture medium. Therefore, these wet extracts were evaporated to dryness and redissolved in dimethyl sulfoxide (DMSO). DMSO was chosen after we determined that the levels of acetone required to dissolve the extracts would be cytotoxic to CHO cells. A preliminary dose-ranging experiment was conducted on each extract using growth inhibition in 24-well culture plates (Linbro) as an index of cytotoxicity (14).

For the cytogenetic assays, wild-type CHO cells were grown in Eagle's minimal essential medium (MEM) (Gibco) supplemented with 7.5% fetal bovine serum (Gibco), sodium pyruvate, nonessential amino acids, penicillin, and streptomycin as previously described (14), with the additional feature that cells were grown attached to 22-mm^2 glass coverslips in 35-mm plastic culture dishes. Cells were treated with the extracts for 1 hr in serum-free medium. Each concentration was tested in triplicate cultures.

Following 26 hr of subsequent incubation in serum-containing medium, coverslip cultures were treated with 1% sodium citrate hypotonic solution for 3 min, fixed in 3:1 methanol:glacial acetic acid for 10 min, air dried, stained in 2% aceto-orcein, and mounted in Permount after dehydration. In each of three replicate cultures (1500 cells), 500 cells per dose level were analyzed for the presence of MN, which represent chromosome fragments excluded from the main nucleus. The in vitro MN assay can be regarded as a rapid test for the detection of chromosomal damage (15).

For the visualization of SCE, cells on coverslips were cultured for 24 hr in medium containing 2×10^{-5} M bromodeoxyuridine (BrdU) and harvested following the addition of 10 mg/mL colchicine for 2 hr. Coverslips were stained with Hoechst 33258 for 10 min and processed as previously described (14). SCEs were scored in 25 cells per culture in each of the three replicate cultures for a total of 75 cells per dose.

In both the SCE and MN assays, cultures were treated as previously described (14), both in the presence and absence of S9 mix prepared from Aroclor 1254-induced, Sprague-Dawley rat liver. All slides were coded so that the observer had no knowledge of the treatment conditions while the slides were scored. The criteria for a positive response were at least a doubling over the background (solvent control) level and indication of a dose-related increase. All extracts were tested at dose ranges including concentrations that resulted in mitotic inhibition.

Results and Discussion

Six sources of raw and treated (chlorinated) drinking water were sampled at the treatment plant sites. The locations were selected to provide a variety of water source types with potentially different spectra of trace organics. Rivers were sources for locations 1 and 2, lakes for locations 3 and 4, and wells for location 6. Location 5 drinking water was obtained from surface water and groundwater that was combined before treatment. The volumes sampled per column ranged from approximately 1200 to 1500 L for treated water and 460 to 1484 L for the raw water. Clogging of the columns with sediment or discontinuous pumping resulted in low sample volumes of raw water from some locations.

Since the mutagenicity data obtained are extensive, only representative responses will be shown in detail. Figure 1 shows the Salmonella test data from location 2 for strain TA 100, the most responsive strain in this
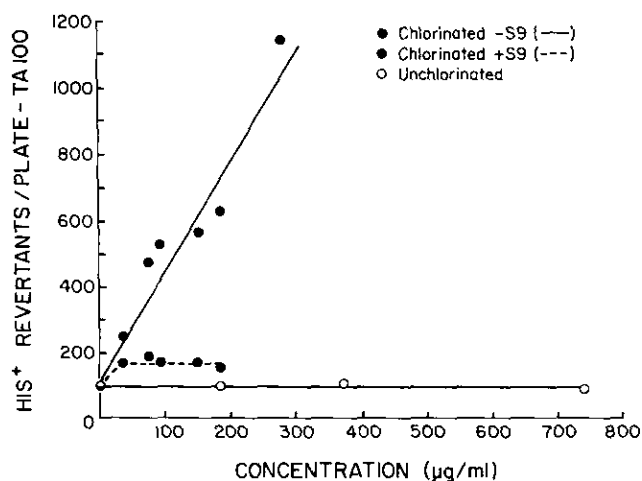
FIGURE 1. Effect of drinking water extracts from location 2 on mutagenicity in Salmonella. To calculate the amounts of extract per plate, the amount per milliliter should be multiplied by 2.7.

case. The extract of raw water had no effect; whereas, the extract of chlorinated water showed a pronounced dose-related effect. This response was virtually eliminated in the presence of S9 mix. The Salmonella data shown in this report are taken from samples evaporated to dryness and dissolved in acetone; however, the wet samples (data not shown) caused essentially the same responses for all samples.

Figure 2 shows the CHO data for the same location (no. 2) as above. No effect was seen when cells were treated in the presence of S9 mix. For the raw water extract, the MN test data exhibit a small dose-related increase for the concentrations tested in the absence of S9 mix. The response was greater in the chlorinated sample, both in terms of the maximum effect and in terms of the lowest effective concentrations. Unfortunately, because of abnormally high values in the solvent controls, the apparent responses would not be considered positive. The fluctuation of solvent control values, which obfuscates such an apparent positive response, is a drawback of the in vitro MN assay as performed here. It is not known if the problem results from a property of the cell line, the culture conditions, or a combination of both. However, the advantages of this assay in terms of cost and utility make it worthy of further investigation. Using pooled negative control values for the study, it is possible to compensate partially for this problem. The mean control values corresponding to -S9 data in Figure 2 are 2.7 micronuclei/100 cells for the unchlorinated and 2.6 micronuclei/100 cells for the chlorinated sample. Accordingly, the maximum response for the chlorinated sample would be greater than twice the control level, and the dose-response would then be considered positive by the criteria stated in the "Materials and Methods" section. The response for the unchlorinated sample would still be considered negative. Similar comparisons for all other samples did not increase the number of positive responses.
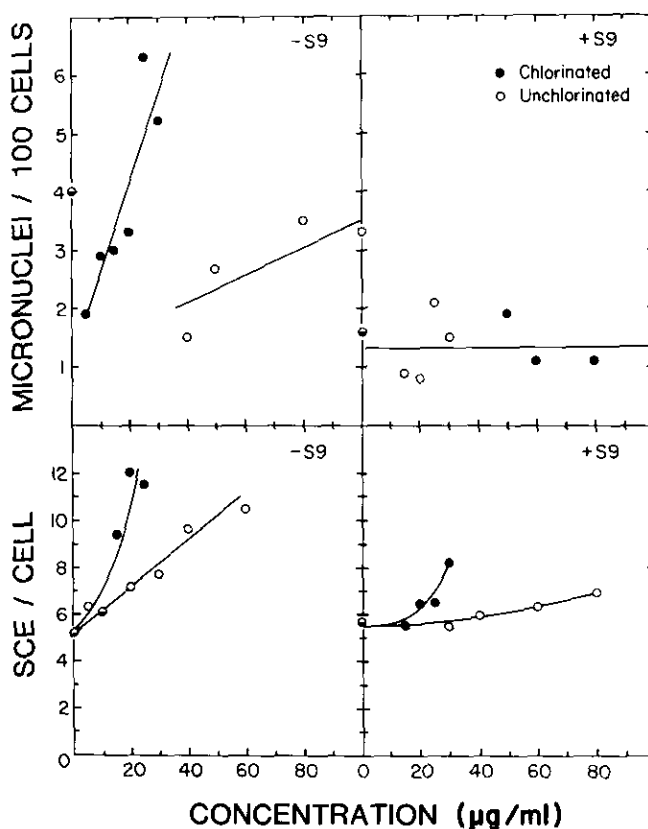

FIGURE 2. Effect of drinking water extracts from location 2 on the induction of MN and SCE in CHO cells.

A positive SCE response occurred with the raw water extract from location 2 in the absence of S9 mix (Fig. 2), in contrast to the findings with the Salmonella assay (Fig 1). This effect was greater in the chlorinated water extract. In the presence of S9 mix, a noticeable but reduced response which is not positive using the criteria applied in this study, was detected for the chlorinated water extract. No effect was seen for the raw water extract treated in the presence of S9 mix.

The data for all six sampling locations are summarized in Figures 3 and 4 according to two parameters: the maximum-fold increase over background and the lowest dose (liter equivalents) tested that induced a doubling over background. The former criterion, although not a measure of potency that is a function of dose, does provide insight into relative responses of the different assays. The latter doubling-dose criterion is a relatively conservative index, but it has been used almost universally as an indicator of biological activity. The data shown in Figures 3 and 4, unless indicated otherwise, are from tests performed without the addition of S9 mix to the treatment mixture, and the Salmonella data are for strain TA 100.

All six raw water extracts gave a negative response in the Salmonella assay; however, all extracts of chlorinated water induced maximum responses greater than twice the background levels. Maximum responses were
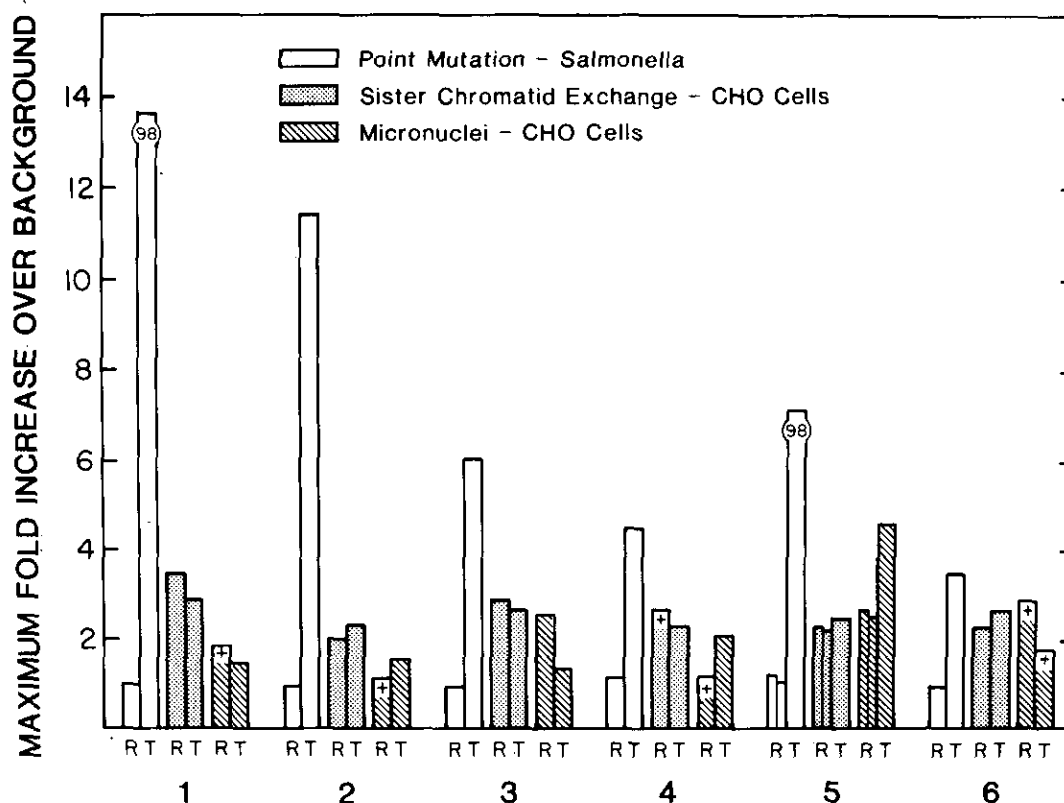
FIGURE 3.   Spectrum of maximum responses in Salmonella and CHO cells after treatment with drinking water extracts from the numbered
sources. Response units are the same as in Figs. 1 and 2. R = raw water, T = treated water. All Salmonella data are for strain TA 100
unless indicated by the designation "98" for TA 98. All data are for assays conducted without S9 mix except as indicated with a "+" for
results of conducted with S9 mix. The split columns for raw water from location 5 indicate groundwater and surface sources, respectively.

observed in all cases without S9 mix, and in two cases, strain TA 98 was more responsive (locations 1 and 5).

For the control (blank) cartridge referred to in the "Materials and Methods" section, negligible material was recovered. Accordingly, only the Salmonella test was performed in TA 100 on a limited basis. The response was not different from the background level, suggesting that the solvent did not interact with the material in the column to generate artificial results.

In the mammalian cell assays, the difference in responses between raw and treated water extracts was not nearly as discrete as in Salmonella. In most cases, in contrast to results shown in Figure 2, little difference was found between the raw and treated samples in terms of the magnitude of responses, particularly for SCE. Although the maximum responses observed in the MN assay were clearly greater for samples from locations 4 and 5, similar high values for SCE were not observed for the same locations. Unlike the Salmonella assay for all locations, the MN assay gave clear decreases in maximum response after chlorination for locations 3 and 6. A further difference between the Salmonella responses and those with mammalian cells was that some of the maximum mammalian cell responses were observed with the addition of S9 mix to the treat-

ment mixture (locations 1, 2, 4, and 6). Comparison of the MN data in Figure 2 with the corresponding responses in Figure 3 for location 2 readily demonstrates the problem created by the high background level as discussed above.

Figure 4 compares the potency of responses seen in Figure 3. Care must be exercised in interpreting Figure 4, since the lower the dose (and the shorter the histogram bar) that induces at least a doubling over the solvent control value, the more potent the response. This parameter is not necessarily directly related to the maximum response seen in Figure 3, although in many cases it is.

As in Figure 3, the consistent effect of chlorination of the Salmonella responses can be seen in Figure 4. For SCE, differences between raw and treated samples are much more evident in this figure than in Figure 3; the potency of the chlorinated sample is higher for locations 2, 3, 5, and 6, but lower for locations 1 and 4. Interestingly, the maximum SCE response for the raw sample from location 4 was found with in vitro metabolic activation (Fig. 3), but the same sample induced a doubling at a lower dose without metabolic activation.

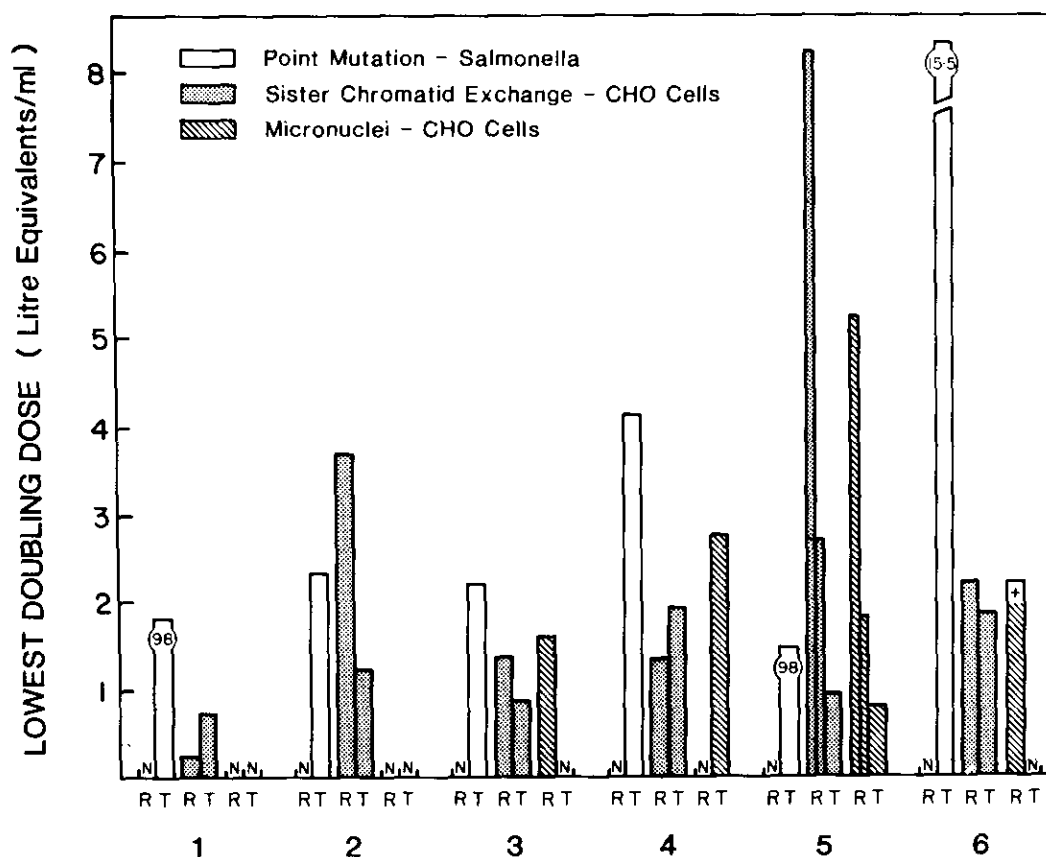The direction of the MN responses tends to coincide with the SCE data in most cases (for example, locations

FIGURE 4.   Relative potency of drinking water extracts from the numbered sources in tests on Salmonella and CHO cells. See Fig. 3 for explanation of common symbols. Response units are the same as in Figs. 1 and 2. N = response less than a doubling over background.

2 through 6), if negative responses are included. However, the raw water sample from location 6 required metabolic activation for maximum potency in the MN assay. This finding suggests that the groundwater source in location 6 contained a different spectrum of trace organics than the surface water sources. It might be expected that if the groundwater sources contained lower levels of trace organics than the surface water sources, then this difference would be reflected by lower responses of the tests to extracts derived from groundwater. Accordingly, the lowest potencies observed for raw water were from groundwater sources (locations 5 and 6), but chlorination of the combined groundwater and surface water at location 5 produced among the most potent responses for all assays.

While the samples collected for this study were being tested for genotoxic effects, parallel chemical analysis (12) revealed that all samples contained diethylhexyl phthalate (DEHP) that was introduced accidentally by the contract laboratory as the concentrates were being prepared. Investigation revealed that the source of the contamination was a plastic connecting sleeve used in error in the cartridge elution system. To determine if this contamination could have influenced the results obtained, experiments were conducted in which hexane/

acetone extracts of the plastic sleeve were added to drinking water extracts. Results indicated that the induction of SCE in CHO cells was not altered by the addition of the plastic extract (data not shown). In subsequent experiments the sampling and extraction procedures were carried out on local drinking water duplicating the conditions that led to the contamination. This column was paired with one on which the correct procedure was used. Extracts collected were then tested in Salmonella and in CHO cells for SCE. Results using contaminated samples (verified as DEHP by gas chromatography) were not different from those using uncontaminated samples, either in terms of gene mutation (Fig. 5) or SCE (Table 1). Earlier samples from this source induced both gene mutation in Salmonella and SCE in CHO cells; however, the data in Table 1 show no appreciable increase in SCE caused by inherent cytotoxicity, although gene mutation was detectable (Fig. 5).

The in vitro bioassays used in this study are indicators of biological effects of the chlorination of drinking water. Levels of over 80 target organics in identical samples analyzed in the companion study (12) showed no aggregate differences, based on chemical class, between raw and chlorinated water extracts. Previously, an attempt
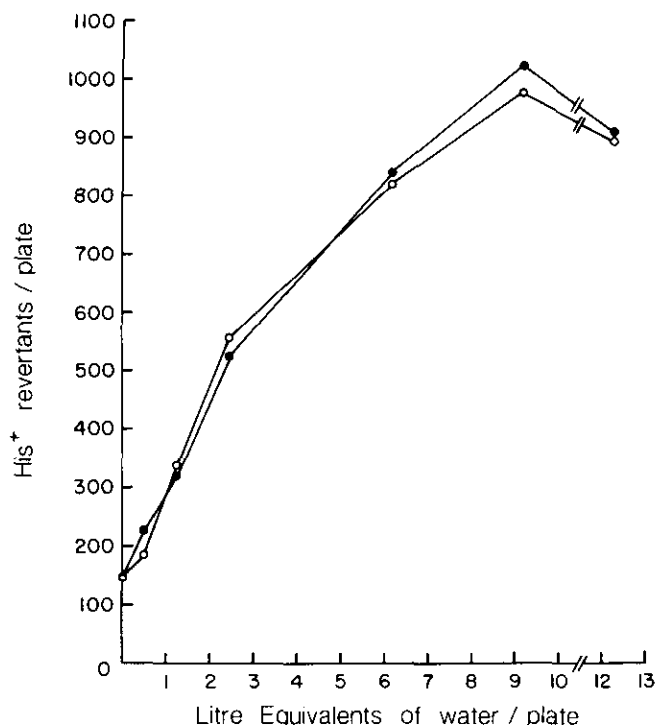
FIGURE 5. Effect of plastic tubing extract contamination introduced during elution of drinking water organics from XAD-2 column on mutagenicity in Salmonella strain TA 100: (○) uncontaminated; (●) contaminated.

to correlate levels of Salmonella mutagenicity with levels of specific target organics did not permit identification of the mutagens responsible for the activity observed (16).

The data reported in this study permit a number of conclusions to be drawn. This study illustrates the utility of the bioassay approach in studies of the effects of treatment processes on chemical mixtures where the specific chemical changes cannot be readily predicted. The pattern of results suggests that the mammalian assays are sensitive to a different spectrum of organic constituents than the Salmonella test. Furthermore, these results demonstrate the utility of the complementary assay approach for studying complex mixtures. The evidence that drinking water extracts are genotoxic in cultured mammalian cells confirms the results obtained in the Salmonella assay and provides a firmer basis for predicting potential hazard. Ultimately, however, the actual demonstration of hazard must rely on *in vivo* studies.

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Table 1. Effect of plastic tubing extract contamination introduced during elution of drinking water organics from XAD-2 column on SCE in CHO cells.

L Equiv./mL	SCE/Cell[a]	
	Contaminated	Uncontaminated
0 (1% DMSO)	4.2	4.2
0.094	4.3	4.4
0.141	4.5	4.5
0.118	5.8	5.1
0.281	MI[b]	5.1
0.375	MI	MI

[a] Based on 75 cells.
[b] Mitotic inhibition.

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